



Antifungal and anti-biofilm effects of shallot (*Allium ascalonicum*) aqueous extract on *Candida albicans*

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ARTICLE INFO

Article Type:
Original Article

Article History:
Received: 7 April 2018
Accepted: 10 September 2018

Keywords:
Allium ascalonicum
Shallot
Candida albicans
Antifungal activity

ABSTRACT

Introduction: The limited option to combat fungal threat has raised the interest in seeking alternative anti-fungal compounds. This study aimed to determine the antifungal property of aqueous-extracted shallot (*Allium ascalonicum*) against *Candida albicans*, a medically important yeast pathogen. The anti-biofilm property of *A. ascalonicum* aqueous extract was also investigated.

Methods: The antifungal effect of *A. ascalonicum* aqueous extract on *C. albicans* was screened using disc diffusion assay and the minimum inhibitory concentration (MIC) was determined using broth macrodilution. Subsequently, the anti-biofilm property of *A. ascalonicum* aqueous extract was investigated using 2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)-5-[(phenylamino) carbonyl]-2 H-tetrazolium hydroxide (XTT) reduction assay, crystal violet (CV) assay, and microscopic examination.

Results: A zone of *C. albicans* growth inhibition was observed at 10 and 20 g/mL of *A. ascalonicum* aqueous extract. The MIC of *A. ascalonicum* aqueous extract was found at 10 g/mL. Significant differences were found between *A. ascalonicum* aqueous extract -treated and non-treated *C. albicans* in term of biofilm formation activity (XTT assay) and the quantity of biofilm formed (CV assay).

Conclusion: Using a simple and inexpensive extraction procedure, this study revealed the antifungal property of *A. ascalonicum* aqueous extract, which could be useful in exploring novel antifungal compound.

Implication for health policy/practice/research/medical education:

Candidiasis remains as one of the prominent nosocomial infection disease and *Candida* biofilm is one of the main culprits associated with this deadly disease. The result showed the application of *Allium ascalonicum* aqueous extract in anti-*Candida albicans* growth and its biofilm activity. This simple and inexpensive method could be useful for the development of surface disinfectant in tackling the *C. albicans* biofilm issue in the clinical setting.

Please cite this paper as: Ng TS, Looi LJ, Ong BS, Chong PP. Antifungal and anti-biofilm effects of shallot (*Allium ascalonicum*) aqueous extract on *Candida albicans*. J Herbmed Pharmacol. 2018;7(4):236-242. doi: 10.15171/jhp.2018.36.

Introduction

There are about 1.5 million fungal species, where *Candida* is among the 300 pathogenic fungal species that causes ~700 000 cases of infection, annually (1-3). *Candida* can be found as a commensal organism in healthy individuals (4). However, it may exploit the weakness of human defence mechanisms and develop candidiasis in the immunocompromised individual (5). Candidiasis may either be superficial, which involves the skin, hair, nails, oral and vaginal regions, or systemic, which affects the major body organs such as acute disseminated *Candida*

septicemia infection (6-8). Among the pathogenic species of *Candida*, *Candida albicans* appears to be the leading causative agent of candidiasis (>50%) and possesses high mortality rate (9,10). This deadly systemic invasive candidiasis is suggested to be highly correlated with the wide-usage of the medical instruments such as catheter contaminated with *Candida* biofilm (11,12). Statistically, fungus (particularly *C. albicans*) is the third leading cause of catheter-related infections as well as second highest colonization-to-infection with the highest crude mortality (13,14).

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Biofilm is defined as the structural microbial communities that are attached to a surface and encased in a matrix of exopolymeric material (13) and often associated with the pathogenicity of *Candida* (12,15). Known to be the virulent factor of *Candida* species, biofilm serves as the protective barrier for *Candida* species and contributes to boost the antifungal drug resistance. Additionally, biofilm also grants *Candida* species the ability to withstand the attack from host immune system and serves as reservoir site for re-infection (5,13,16). Interestingly, the greater ability of *C. albicans* to form biofilm is linked with its dominance as the leading cause of candidiasis, in comparison to *C. glabrata* and *C. tropicalis* (17).

Despite the severity of candidiasis and fungal infections, fungi are often neglected by the public and funding bodies. In comparison to antibacterial remedies, there are only limited classes of antifungal agents available for physicians to combat *Candida* infections, namely polyenes, azoles, and echinocandins (18) which in turn may complicate the management of patients. In addition, the raising antifungal resistance reported worldwide has worsened the treatment outcomes (10). Therefore, several efforts, such as therapy through research and development of natural products, have been initiated to combat the threat posed by *Candida* species. The use of *Allium ascalonicum* (shallot) in food flavoring is known worldwide. Besides, shallot is also being employed in folk medicine since ancient time where shallot is described as “delicious food that stimulates the appetite during hot weather” (19). Shallot also possesses important medicinal value such as anti-oxidant (19) and anti-microbial property against *Mycobacterium tuberculosis* (21). Extensive efforts such as complex phytochemical analysis have been carried out to identify the potential active compounds, for example ascalin, and ascalonicoside A1/A2/B which may serve as the active antifungal compounds in *A. ascalonicum* (19,22). These studies have highlighted the potential active antifungal compounds found in shallot bulb, however tedious efforts and resources are required for the purification and retrieval of these compounds. Thus, this study aims to use a direct and cost-effective aqueous extraction method to retrieve crude *A. ascalonicum* aqueous extracts and test the antifungal and anti-biofilm properties of the retrieved compounds. The results from this study would be useful for the development of inexpensive and economically sound surface disinfectant, particularly in tackling and managing the biofilms of *C. albicans*, which persists in the clinical settings (13).

Materials and Methods

Preparations of *Allium ascalonicum* aqueous extract

Allium ascalonicum aqueous extract was prepared freshly each time before use. A stock solution of 20 g/mL [weight/volume (w/v)] of *A. ascalonicum* was prepared by grinding 20 g fresh clean shallot and ground in 1 mL of Phosphate

Buffer Saline (PBS). The extract was allowed to stand for 30 minutes at room temperature and centrifuged at 5000 rpm for 10 minutes. Then, the supernatant fluid was filtered through a sterile 0.22 µm membrane filter (Merck Milipore, US). The required concentrations (w/v) were prepared by serial dilution from the stock solutions.

Disc diffusion assay

Preparations of paper disc impregnated with *Allium ascalonicum* extract

The fresh stock solution of 20 g/mL (w/v) of *A. ascalonicum* extract was serially diluted to prepare the serial concentrations of the extract (2.5 g/mL, 5.0 g/mL, 10.0 g/mL and 20.0 g/mL) by using two-fold serial dilution with PBS which served as diluent. Paper discs with 6 mm diameter in size were prepared from ALBET® filter paper (Hahnemühle, Germany) by using the sterile hole puncher. The paper discs were autoclaved at 121°C for 20 minutes for sterilization purpose before used. Then, the prepared paper discs were immersed in respective concentration solution for 10 minutes. Negative control disc was prepared by impregnating the paper disc with 20 µL PBS solution while Clotrimazole (1.6 mg/mL) (Sigma Aldrich) served as positive control. The discs were lifted and left covered at room temperature for 20 minutes before used.

Preparation of *Candida albicans* strains

Candida albicans ATCC 14053 was cultured on Sabouraud Dextrose Agar (SDA) (Becton & Dickinson, USA) and was passaged twice from a single colony to ensure the purity. The identity of *C. albicans* was screened through CHROMagar™ *Candida* (CHROMagar, French), which appeared as green colony. Further test on *C. albicans* colony was performed by amplifying its ITS 1-4 (Internal Transcribed Spacer Regions) through polymerase chain reaction (PCR), which yielded a 530 bp amplicon (23). The *C. albicans* culture with correct identity was then incubated overnight on SDA at 37°C. Five colonies with the diameter larger than 1 mm were picked and suspended in 5 mL of PBS buffer. The yielded suspension was vortexed and cell density was adjusted by adding sufficient PBS buffer to achieve 0.5 McFarland Unit at 600 nm wavelength through microplate reader (BioTek, USA) in order to standardize the cell suspension with 1×10^6 to 5×10^6 cells per mL. The inoculums were spread carefully onto SDA by using sterile glass hockey stick.

Disc diffusion assay

The plant extract disc was applied on the agar and allowed to stand at room temperature for 15 minutes to allow diffusion. Then, the agar plate was incubated at 37°C for 24 hours and the diameter of zone of inhibition was measured.

Broth microdilution

The broth microdilution method was performed as described previously with minor modification (24). A 0.5 McFarland Unit *C. albicans* cell suspension (1×10^6 to 5×10^6 cells per mL) was prepared as described above. Then, 0.1 mL of the prepared cell suspension was mixed with 0.9 mL of Sabouraud Dextrose Broth (SDB) (Becton & Dickinson, USA) for further dilution of cells to 1×10^5 to 5×10^5 cells per mL.

Preparation of diluted *Allium ascalonicum* aqueous extract

A total weight of 40 g fresh *A. ascalonicum* was ground in 1 mL of PBS to prepare a stock solution of 40 g/mL (w/v). The extract was allowed to stand for 30 minutes at room temperature then followed by centrifugation at 5000 rpm for 10 minutes. The supernatant fluid was filtered through a sterile 0.22 μ m membrane filter (Merck Milipore, US). The required concentration, weight/volume (w/v) was prepared by serial dilution from the stock solutions as shown in Table 1.

Broth inoculating

A volume of 1 mL of each extract was pipetted into a 15 mL centrifuge tube, followed by 1 mL of *C. albicans* cell suspension and mixed well. The mixture tube was capped and left at room temperature for 15 minutes, then incubated at 37°C/180 rpm in a shaker incubator (Labnet, USA) for 24 hours. The cell growth activity was observed.

Biofilm formation in 96-well microtitre plate

The biofilm formation of *C. albicans* was performed as described by Pierce et al (25). A loopful of *C. albicans* cells was taken from culture stock of *C. albicans* in SDA and inoculated into 20 mL of SDB media. The culture was then incubated at 37°C/180 rpm for 16 hours in shaking incubator. After that, the cells were centrifuged at 3000 rpm for 5 minutes and the supernatant was removed. The sediment cells were resuspended in 2 mL of PBS for the cell washing purpose. The cells were centrifuged at 3000 rpm for 5 minutes again and the cell washing process was repeated twice. The cells were resuspended and

adjusted to 0.5 McFarland unit with 37°C pre-warmed RPMI 1640 media. Then, 100 μ L of cell suspension was loaded onto the 96-well microtitre plate. Next, 100 μ L of final concentration of plant extract varies from 0 MIC, 0.25 MIC, 0.5 MIC and 1 MIC were pipetted and mixed with cell suspension in respective wells. The 96-well microtitre plate was covered with the lid and sealed with parafilm, followed by incubation for 24 hours at 35°C. After incubation, the media from wells of 96-well microtitre plate was aspirated out carefully in order to prevent disruption of the biofilm formed at the bottom of well. The biofilm of each well was washed by adding 200 μ L of PBS and the washing process was repeated for three times. Then, the plate was placed in invert position with a blotting paper to remove the residual of PBS.

Measurement of Biofilm Activity by using XTT {2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide} reduction assay

The measurement of biofilm activity through XTT assay was performed as described previously (25). Briefly, 4.95 mL of pre-made XTT (0.5 g/L) was thawed and 50 μ L of menadione was added to thawed XTT. 100 μ L of XTT/menadione was added to washed-biofilm in the wells of 96-well microtitre plate. Then, the plate was wrapped with aluminium foil and incubated in dark at 37°C for 3 hours. After that, 80 μ L of supernatant was transferred to a new 96-well microtitre plate and the plate was read by using microtitre reader (BioTek, USA) at the wavelength of 490 nm.

Quantification of biofilm by using crystal violet assay

The biofilm formed was quantified as described previously (26). Generally, the washed-biofilm was fixed by adding 100 μ L of methanol to each well for 15 minutes. Then, the supernatant was removed and the plate was left for air-dried. Subsequently, crystal violet (CV) solution was prepared by adding 1 mL of 90% CV in 50 mL distilled water and 100 μ L of it was added to each well and left for 20 minutes. The excess CV was washed away under running tap water. A total of 150 μ L of 33% acetic acid was

Table 1. Preparation of serial concentrations of *Allium ascalonicum* extract

Concentration (g/mL)	Stock solution at 40 g/mL (mL)	PBS (mL)	Mixed with SDB	Final concentration yielded (g/mL)
0	0	1		0
4	0.1	0.9		2
8	0.2	0.8		4
12	0.3	0.7		6
16	0.4	0.6		8
20	0.5	0.5	1 mL of extract + 1 mL of <i>C. albicans</i> suspension in SDB	10
24	0.6	0.4		12
28	0.7	0.3		14
32	0.8	0.2		16
36	0.9	0.1		18
40	1	0		20

added to each well to release the bounded CV. Then, the plate was read by using microtitre reader (BioTek, USA) at the wavelength of 590 nm.

Biofilm formation in 6-well microtitre plates

The suspension of *C. albicans* cell in RPMI1640 medium was prepared as described above. A volume of 2.5 mL of cell suspension was pipetted onto 6-well microtitre plates. Next, 2.5 mL of *A. ascalonicum* extract with 4 different final concentrations (0 MIC, 0.25 MIC, 0.5 MIC and 1 MIC) were added into the cell suspension. The 6-well microtitre plate was covered with lid and incubated for 24 hours at 35°C.

Microscopic observation of biofilm formation

After 24 hours, 6-well microtitre plate was viewed and examined under inverted light microscope. Photographs were taken for each concentration of *A. ascalonicum* extract.

Statistical analysis

Data analyses were performed using IBM SPSS Statistics (Version 23.0). The normality of the data was accessed using Shapiro-Wilk normality test. Results were analyzed using Kruskal-Wallis and Bonferroni correction test to determine the differences between treated and untreated groups and *P* values < 0.05 were considered as statistically significant.

Results

Disc diffusion assay and broth macrodilution assay

In this study, the anti-*C. albicans* effect of *A. ascalonicum* aqueous extract was screened by using disc diffusion assay. A clear zones of inhibition, 1.2 cm and 1.5 cm were observed at the 10 g/mL and 20 g/mL of *A. ascalonicum* aqueous extract, respectively (Figure 1). Broth macrodilution found that the minimum inhibitory concentration (MIC), the lowest concentration of *A. ascalonicum* aqueous extract that inhibited the growth of

C. albicans was 10 g/mL (Table 2).

Biofilm formation assay

The Shapiro-Wilk test has indicated that the data generated for both XTT and CV assays were not normally distributed ($P < 0.05$). Therefore, the non-parametric statistical tests, Kruskal-Wallis and Bonferroni correction tests were applied in this study. Through the XTT reduction assay, significant reduction in biofilm forming activity was observed when biofilm formation of *C. albicans* was challenged with *A. ascalonicum* extract at 1 MIC and 2 MIC, in comparison to untreated biofilm (Figure 2). Besides, CV assay found that the amount of biofilm formed by *C. albicans* was observed to be reduced significantly with the treatment of *A. ascalonicum* extract at 1 MIC and 2 MIC (Figure 3). The microscopic examination of *C. albicans* biofilm treated with *A. ascalonicum* extract was carried out by using inverted light microscope at 40X magnification to examine the anti-biofilm effect of *A. ascalonicum* extract, qualitatively. The results showed

Table 2. Summary of the Minimum Inhibitory Concentration (MIC) of *Allium ascalonicum* aqueous extracts against *Candida albicans*

Plant Extracts Concentration, g/mL	<i>Candida albicans</i> growth
Negative Control	Resistant
2	Resistant
4	Resistant
6	Resistant
8	Resistant
10	Susceptible
12	Susceptible
14	Susceptible
16	Susceptible
18	Susceptible
20	Susceptible
Positive Control (Clotrimazole)	Susceptible

The growth of *C. albicans* was inhibited by 10 g/mL of *A. ascalonicum*.

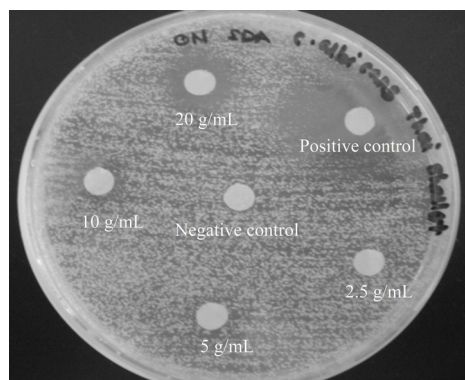


Figure 1. The disc diffusion assay showed 10 g/mL and 20 g/mL of *Allium ascalonicum* aqueous extracts inhibited the growth of *Candida albicans*.

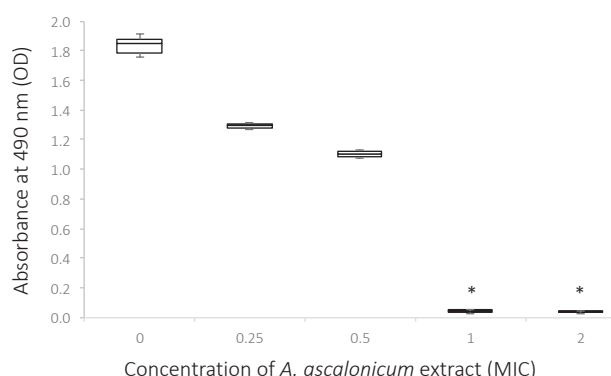


Figure 2. Determination of the effect of *Allium ascalonicum* aqueous extract on *Candida albicans* biofilm formation activity using XTT Assay. Box-plot shows the biofilm formation activity under different concentration of treatment after incubation for 24 hours, at 490 nm by using XTT reduction assay. A significant reduction (*) of biofilm formation activity was observed at 1 MIC and 2 MIC (P value < 0.05).

that the biofilm density of *C. albicans* was reduced with the increasing concentration of *A. ascalonicum* extract, compared to biofilm formation without any treatment (Figure 4).

Discussion

Candidiasis is a type of opportunistic infection in human caused by *Candida* species. *C. albicans* is the major etiological agent which constitutes 44.2%, among 1114 clinical isolations in Malaysia (27). In past 2 decades, the prevalence of candidiasis has risen and this led to the increase in the usage of antifungal therapies such as azole drug. With the extensive usage of azole drug, the problem with the development of resistance toward azoles by *Candida* species has also risen with both primary and secondary resistance (28). Furthermore, the persistence of *Candida* species biofilm on medical devices surfaces such as indwelling catheter has also contributed to the rising number of invasive candidiasis cases (12). The formation of biofilm is also linked to the enhanced capability of *Candida* to resist antifungal (29), which leads to the challenge in the treatment of candidiasis. Thus, the purpose of this study was to evaluate antifungal and anti-biofilm properties of the aqueous extract from *A. ascalonicum* against the predominance *Candida* species, *Candida albicans* which can be applied as an economic surface disinfectant or novel antifungal compound. The disc diffusion assay suggested that the effective concentration of *A. ascalonicum* aqueous extract to inhibit the growth of *C. albicans* was at 10 g/mL (Figure 1) and broth microdilution assay was determined the MIC of *A. ascalonicum* aqueous extract to be 10 g/mL (Table 2), where the growth of *C. albicans* was completely stunted. This is in line with the previous study conducted by Mahmoudabadi and Nasery (30) where fresh crude shallot juice (*A. ascalonicum* Linn. (Liliaceae)) presented with antifungal activity against *C. albicans*, as well as saprophytic fungi and dermatophytes. However, the

MIC was not comparable as the authors designated the concentration of *A. ascalonicum* aqueous extract in the form of percentage, rather than in weight per volume. Besides, several studies have also highlighted the potential antifungal effect of *A. ascalonicum* in combating *C. albicans* (31,32), at mg/mL level. Notably, the extraction methods in both studies were different from this study, where *A. ascalonicum* has undergone complex and time-consuming extraction procedure while current study applied the direct yet simple (<1-hour procedure) aqueous method to extract the antifungal compound from *A. ascalonicum*. Followed by the susceptibility test, the potential of *A. ascalonicum* extract to combat the biofilm of *C. albicans* was evaluated through both XTT assay and CV assay. XTT is a yellow salt that is reduced by mitochondrial dehydrogenase of metabolically active yeast cell (33) and is employed in quantitating the biofilm formation activity (34) while CV assay is a basic dye which binds to negatively charged extracellular molecule in mature biofilm (35) and CV assay can be applied in measuring the amount of biofilm formed (25,26). In this study, both XTT assay and CV assay showed that *A. ascalonicum* aqueous extract exhibited an inhibitory effect on biofilm formation of *C. albicans* (Figures 3 and 4). Moreover, the increased in the concentration of *A. ascalonicum* extract has resulted in a greater reduction of biofilm formation activity and biofilm density (Figures 2 and 3). These observations were further verified with the microscopic examination

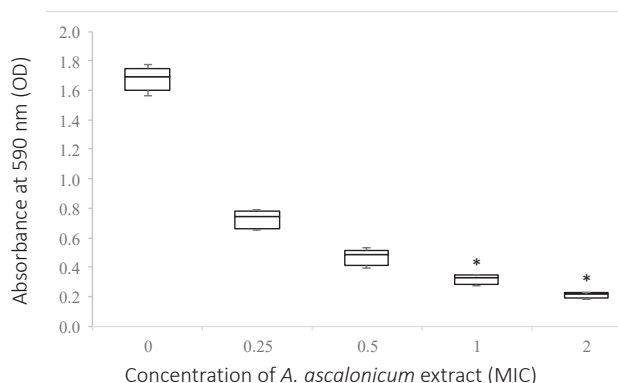


Figure 3. Determination of the effect of *Allium ascalonicum* aqueous extract on *Candida albicans* biofilm formation using CV assay. Box-plot shows the amount of biofilm formed under different concentration of treatment after incubation for 24 hours, at 590 nm by using CV assay. A significant reduction (*) of biofilm formed was observed at 1 MIC and 2 MIC (P value < 0.05).

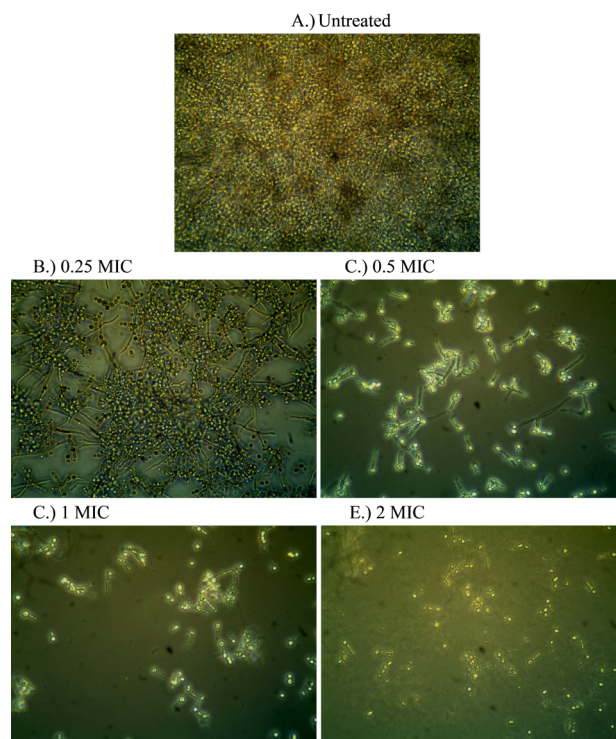


Figure 4. Biofilm formation observed after 24 hours with respective treatment applied. A.) without treatment B.) 0.25 MIC, C.) 0.5 MIC, D.) 1 MIC and E.) 2 MIC at 40X magnification. A reduction of activity was observed with increasing concentration of treatment.

as biofilm density decreased with the treatment of *A. ascalonicum* extract (Figure 4).

Conclusion

In short, through a simple and cost-effective aqueous extraction method, this study revealed the potential of *A. ascalonicum* to inhibit the growth of *C. albicans* at the concentration of 10 g/mL as well as to inhibit the biofilm formation activity of *C. albicans*. The results from this study are beneficial for the development of economically-sound surface disinfectant which could be useful in managing the *Candida* biofilm contamination.

Acknowledgments

This paper was derived partly from B.Sc theses of Tzu Shan Ng and Ban Shui Ong. The authors are in debt for the financial support provided by the Department of Biomedical Sciences, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia.

Authors' contributions

TSN and BSO performed the experiment. TSN and LJL wrote the manuscript. TSN, BSO and PPC designed and conceptualized the experiment designed. All read and confirmed final version of the manuscript for publication.

Conflict of interests

The authors declared no competing interests.

Ethical considerations

Ethical issues (including plagiarism, misconduct, data fabrication, falsification, double publication or submission redundancy) have been completely observed by the authors.

Funding/Support

Final year student project allocation, Department of Biomedical Sciences, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia.

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